Supporting Information:

Discovery of a Potent, Selective and Orally Bioavailable Acidic 11β-Hydroxysteroid Dehydrogenase Type 1 (11β-HSD1) Inhibitor: The Discovery of 2-[(*3S*)-1-[5-(cyclohexylcarbamoyl)-6-propylsulfanyl-pyridin-2-yl]-3-piperidyl]acetic acid (AZD4017).

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Contents:

Synthesis of Intermediates:

2,6-Dichloro-*N*-cyclohexyl-pyridine-3-carboxamide (9)

To a solution of 2,6-dichloronicotinic acid **8** (5.005 g, 26.18 mmol) in CH_2Cl_2 (60 mL) was added a few drops of DMF followed by the addition dropwise of oxalyl chloride (2.27 mL, 26.18 mmol). The reaction was stirred at room temperature for two hours until gas evolution had ceased. The solvent was evaporated under reduced pressure to give an oil. CH_2Cl_2 (60 mL) was added and the reaction mixture was cooled in an ice bath. Cyclohexylamine (5.98 mL, 52.36 mmol) was added slowly keeping the temperature below 15°C. The reaction was stirred at room temperature overnight. The reaction mixture was extracted in CH_2Cl_2 and washed with sat $NaHCO_3$ (30 mL), water (30 mL) and brine. The solvent was evaporated under reduced pressure to give a brown/red solid. It was recrystalised in hexane/ethyl acetate and filtered to give a white solid (6.986 g, 98%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.07 – 1.36 (5H, m), 1.52 – 1.61 (1H, m), 1.66 – 1.75 (2H, m), 1.79 – 1.88 (2H, m), 3.66 – 3.77 (1H, m), 7.64 (1H, d, *J* = 7.9), 7.94 (1H, d, *J* = 7.9), 8.52 (1H, d, *J* = 7.8); LRMS m/z (M^+ + H) 273.

6-Chloro-*N*-cyclohexyl-2-propylsulfanyl-pyridine-3-carboxamide (10)

To a solution of propane thiol (3.0 mL, 32.9 mmol) in DMF (25 mL) was added slowly a solution of 1M NaHMDS in THF (33 mL, 33.00mmol). The mixture was stirred for ten minutes at room temperature and then added slowly to a solution of 2,6-dichloro-*N*-cyclohexyl-pyridine-3-carboxamide **9** (8.95 g, 32.89 mmol) in DMF (50 mL). The reaction was stirred at room temperature for two hours. The reaction was stopped and the majority of the THF and DMF was evaporated. The product was extracted with CH_2Cl_2 (150 mL), washed with water (2 x 25 mL) and brine (25 mL). The solution was dried over $MgSO_4$ and evaporated under reduced pressure to give a slightly pink solid. The solid was triturated in hexane to give a white solid (7.84 g, 76%). ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.98 (3H, t, *J* = 7.4), 1.12 – 1.29 (3H, m), 1.31 – 1.41 (2H, m), 1.52 – 1.61 (1H, m), 1.62 – 1.73 (4H, m), 1.91 – 2.01 (2H, m), 3.04 (2H, t, *J* = 7.4), 3.87 – 3.98 (1H, m), 6.12 – 6.30 (1H, m), 6.96 (1H, d, *J* = 8.0), 7.72 (1H, d, *J* = 8.0); LRMS m/z (M^+ + H) 313.

6-Chloro-*N*-cyclohexyl-2-propoxynicotinamide (12a)

2,6-Dichloro-*N*-cyclohexylnicotinamide **9** (273 mg,1 mmol) was stirred in 1-propanol (4 mL) then bis-sodium hexamethyldisilylamide 1.0 M in THF (1.1 mL,1.1 mmol) was added. The mixture was subjected to microwave heating at 150 °C (Biotage Initiator 300W) for 2 hours. The solvent was removed *in vacuo* and the residue taken up in dichloromethane (20 mL) washed with water (20 mL), brine (20 mL) and dried over $MgSO_4$ then filtered and evaporated. Chromatography (SiO_2) eluting with ethyl actate/isohexane 0-40% gave 6-chloro-*N*-cyclohexyl-2-propoxynicotinamide as a white solid (211 mg, 71%). ^{1}H NMR (400 MHz, DMSO-d_6) δ1.00 (3H, t, *J* = 7.4), 1.17 – 1.39 (5H, m), 1.54 – 1.57 (1H, m), 1.64 – 1.88 (6H, m), 3.76 – 3.79 (1H, m), 4.30 (2H, t, *J* = 6.4), 7.20 (1H, d, *J* = 7.8), 7.97 (1H, d, *J* = 7.8), 8.09 (1H, d, *J* = 7.8); LRMS m/z (M^+ + H) 297.

6-Chloro-*N*-cyclohexyl-2-(propylamino)nicotinamide (12b)

A mixture of 2,6-dichloro-*N*-cyclohexylnicotinamide **9** (273 mg,1 mmol), *n*-propylamine (91 μL, 1.1 mmol), potassium carbonate (345 mg, 2.5 mmol) in butyronitrile (4 ml) was sealed in a microwave tube and heated (Biotage initiator 300 W) at 150 °C for 1 hour. The reaction was diluted with water (25 mL) and extracted with dichloromethane (2 x 25 mL). The combined extracts were dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. Chromatography (SiO_2) eluting with ethyl acetate/isohexane 0-30% gave 6-chloro-2(propylamino)-*N*-cyclohexylnicotinamide as a white powder (180 mg, 61%). ^{1}H NMR (400.13 MHz, DMSO-d_6) δ 0.91 (3H, t, *J* = 7.4), 1.05 – 1.35 (5H, m), 1.49 – 1.64 (3H, m), 1.66 – 1.84 (4H, m), 3.27 – 3.31 (2H, m), 3.70 (1H, m), 6.59 (1H, d, *J* = 8.0), 7.95 (1H, d, *J* = 8.0), 8.26 (1H, d, *J* = 7.7), 8.68 (1H, t, *J* = 5.3); LRMS m/z (M^+ + H) 296.

6-Chloro-*N*-cyclohexyl-2-[methyl(propyl)amino]nicotinamide (12c)
Prepared according to the procedure of **12b** from 2,6-dichloro-*N*-cyclohexylnicotinamide **9** and *N*-methyl propylamine in 60% yield;
^{1}H NMR (400 MHz, DMSO-d_6) δ 0.81 (3H, t, *J* = 7.4), 1.10 – 1.34 (5H, m), 1.48 – 1.60 (3H, m), 1.69 – 1.73 (2H, m), 1.77 – 1.85 (2H, m), 2.89 (3H, s), 3.30 – 3.40 (2H, m), 3.63 – 3.67 (1H, m), 6.64 (1H, d, *J* = 7.6), 7.42 (1H, d, *J* = 7.6), 8.32 (1H, d, *J* = 7.9); LRMS m/z (M^+ + H) 310.

5-Ethyl 1-methyl-4-(1-aminopentylidene)pent-2-enedioate (16)
Ethyl -3-aminohept-2-enoate (7.8 g, 45.2 mmol) was stirred in toluene (80 mL) then methyl propiolate (4.86 mL, 54.73 mmol) was added and the reaction was stirred under N_2 at 100 °C for 96 hours. The solvent was evaporated to give an orange oil. Chromatography (SiO_2) eluting with ethyl acetate/isohexane (20-40%) gave 5-ethyl 1-methyl -4-(1-aminopentylidene) pent-2-enedioate (9.5 g, 81%) as a yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.97 (3H, t, *J* = 7.2), 1.27 – 1.51 (5H, m), 1.57 – 1.68 (2H, m), 2.49 – 2.58 (2H, m), 3.74 (3H, s), 4.26 (2H, q, *J* = 6.9), 6.21 (1H, d, *J* = 15.3), 7.67 (1H, d, *J* = 15.3); LRMS m/z (M^+ + H) 256.

Ethyl 2-butyl-6-oxo-1,6-dihydropyridine-3-carboxylate (17)
5-Ethyl 1-methyl-4-(1-aminopentylidene) pent-2-enedioate **16** (2.0 g, 7.8 mmol) and sodium *tert*-butoxide (100 mg, 1.0 mmol) were stirred in NMP (20 mL). The solution was heated at 180 °C for 4 hours giving a dark solution. On cooling the reaction was diluted with ice/water (50 mL) and the resulting precipitate was filtered and washed with water (10 mL) and dried to give ethyl 2-butyl-6-oxo-1,6-dihydropyridine-3-carboxylate (1.35 g, 78%) as a grey powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.90 (3H, t), 1.28 (3H, t, *J* = 7.2), 1.24 – 1.39 (5H, m), 1.50 – 1.58 (2H, m), 2.86 – 2.94 (2H, m), 4.22 (2H, q, *J* = 7.1), 6.21 (1H, d, *J* = 9.7), 7.82 (1H, d, *J* = 9.7), 11.97 (1H, s); LRMS m/z (M^+ + H) 224.

Ethyl 2-butyl-6-chloronicotinate (18)
Ethyl 2-butyl-6-oxo-1,6-dihydropyridine-3-carboxylate **17** (450 mg, 2.02 mmol) was stirred in phosphorous oxychloride (10 mL, 30.5 mmol) and heated to 120 °C for 2 hours giving a clear brown solution. The reaction was evaporated and the residue was taken up in EtOAc (25 mL), washed with water (25 mL), saturated brine (25 mL) then dried over $MgSO_4$ filtered and evaporated. Chromatography of the residue (SiO_2) eluting with ethyl acetate/isohexane (10-30%) gave ethyl 2-butyl-6-chloronicotinate (395 mg, 81%) as a clear oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.91 (3H, t, *J* = 7.3), 1.30 – 1.39 (5H, m), 1.58 – 1.65 (2H, m), 2.99 – 3.05 (2H, m), 4.34 (2H, q, *J* = 7.2), 7.48 (1H, d, *J* = 8.3), 8.17 (1H, d, *J* = 8.3); LRMS m/z (M^+ + H) 242.

2-Butyl-6-chloronicotinic acid (19)
Ethyl 2-butyl-6-chloronicotinate **18** (395 mg, 1.63 mmol) was stirred in methanol (10 mL) and 2M sodium hydroxide (2 ml, 4 mmol) was added. The solution was stirred at room temperature for 16 hours. The solvent was evaporated and the residue was taken up in ice/water (10 mL) and acidified with 2M HCl. The solution was extracted with dichloromethane (2 x 15 mL) and the combined extracts were dried over $MgSO_4$, filtered and evaporated. Trituration with isohexane gave 2-butyl-6-chloronicotinic acid (300 mg, 86%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.90 (3H, t, *J* = 7.3), 1.29 – 1.38 (2H, m), 1.58 – 1.66 (2H, m), 3.02 – 3.10 (2H, m), 7.44 (1H, d, *J* = 8.2), 8.17 (1H, d, *J* = 8.2), 13.41 (1H, s); LRMS m/z (M^+ + H) 214.

2-Butyl-6-chloro-*N*-cyclohexylnicotinamide (20)
2-Butyl-6-chloronicotinic acid **19** (300 mg, 1.4mmol) and 1-hydroxybenzotriazole (209 mg, 1.54 mmol) were stirred under nitrogen in dichloromethane (20 mL). To this was added triethylamine (431 μL, 3.1 mmol) followed by EDAC (295 mg, 1.54 mmol). After 5 minutes cyclohexylamine (161 μL, 1.4 mmol) was added and the reaction was stirred at room temperature for 16 hours. The reaction was washed with saturated $NaHCO_3$ (25 mL), 1M HCl

(25 mL), water (25 mL), brine (25 mL) and dried over $MgSO_4$ then filtered and the solvent removed *in vacuo*. Trituration with isohexane gave 2-butyl-6-chloro-N-cyclohexylnicotinamide (310 mg, 75%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.93 (3H, t, *J* = 7.3), 1.13 – 1.44 (7H, m), 1.59 – 1.71 (3H, m), 1.73 – 1.82 (2H, m), 1.84 – 1.93 (2H, m), 2.80 – 2.88 (2H, m), 3.72 – 3.85 (1H, m), 7.44 (1H, d, *J* = 8.0), 7.76 (1H, d, *J* = 8.0), 8.45 (1H, d, *J* = 7.9); LRMS m/z (M^+ + H) 295.

Biological Protocols:

Measurement of 11β-HSD2 activity: 11β-HSD2 catalyses the conversion of cortisol to cortisone. The compounds were incubated with a mixture consisting of 11β-HSD2 recombinant enzyme in 1 mM DTT, NAD (Roche Diagnostics, 2.5 mM) and cortisol (Sigma, Poole, Dorset, UK, 1 mM, 0.625 μM) in a total volume of 50 μl in 384 well plates. Assay plates were read 40 min post cortisol addition on a fluorescent plate reader (Envision) with signal excitation 340 nm (25 nm band width) and emission 460 nm.

Measurement of 17β-HSD1 activity: 17β-HSD1 catalyses the conversion of estrone to estradiol. The assay incubation was carried out in borosilicate glass tubes consisting of estradiol (Sigma, Poole, Dorset, UK, 160 nM), glucose-6-phosphate (Roche Diagnostics, 1 mM), NADPH (Roche Diagnostics, 100 μM), glucose-6-phosphate dehydrogenase (Roche Diagnostics, 12.5 μg/ml), EDTA (Sigma, Poole, Dorset, UK, 1 mM), assay buffer (K_2HPO_4/KH_2PO_4, 100 mM) pH 7.5, recombinant 17β-HSD1 (1.5 μg/ml) plus test compound containing 1 μCi 3H estrone (Perkin Elmer). The compounds were incubated for 20 minutes at room temeprature and the reaction stopped by the addition of ethyl acetate. Radiolabelled steroids were separated using reversed phase HPLC, Agilent 1200 HPLC using a Zorbax Eclipse XDB-C18 5 μm column, 150 x 4.6 mm (Crawford Scientific, Lanarkshire, UK) with acetonitrile:H_2O (50:50) at flow rate of 1 ml/min. Radioactivity measured using a flow scintillation analyser (Radiomatic series 500TR, Perkin Elmer Analytical Instruments) with FLO-ONE software.

Measurement of 17β-HSD3 activity: 17β-HSD3 catalyses the conversion of androstenedione to testosterone. The assay incubation was carried out in deep well plates tubes consisting of glucose-6-phosphate (Roche Diagnostics, 1mM), NADPH (Roche Diagnostics, 100 μM), glucose-6-phosphate dehydrogenase (Roche Diagnostics, 12.5 μg/ml), EDTA (Sigma, Poole, Dorset, UK, 1 mM), assay buffer (K_2HPO_4/KH_2PO_4, 100 mM) pH 7.5, recombinant 17β-HSD3 (1.5 μg/ml) plus test compound containing 1 μCi ^{3}H androstenedione (Perkin Elmer). The compounds were incubated for 90 minutes at 37 °C and the reaction stopped by the addition of 1 mM glycerrhetinic acid. Radiolabelled steroids were separated using reversed phase HPLC, Agilent 1200 HPLC using a Zorbax Eclipse XDB-C18 5 μm column, 150 x 4.6 mm (Crawford Scientific, Lanarkshire, UK) with acetonitrile:H_2O (35:65) at flow rate of 2 ml/min. Radioactivity measured using a flow scintillation analyser (Radiomatic series 500TR, Perkin Elmer Analytical Instruments) with FLO-ONE software.

Crystallography:

Initial attempts to produce structures of human 11β-HSD1 in complex with compounds using literature constructs were unsuccessful which resulted in a protein engineering campaign aimed at producing a soakable system in order to provide structural support for the drug discovery process. Over 40 constructs were designed and assessed for overexpression and crystallisation. The preferred construct (11β-HSD1 41D) was broadly based on a human sequence S22-K292 with the following point mutations M179L, L262R, C272S, F278E, M268W. The protein was overexpressed in an E.coli recombinant system and purified using a TEV cleavable 6His *N*-terminal tag. The protein was concentrated in the size exclusion buffer (20 mM Hepes pH 8, 100 mM NaCl, 2 mM TCEP and 10 μM NADP) to an $O.D_{280}$ of 7.0. 2

µl drops of concentrated protein were mixed with well solution (PEG400 30 – 45%, Tris-HCl 100 mM pH 7.6 to 8.5) in a 1:1 ratio and equilibrated as hanging drops (well volume = 500 µl). Apo protein crystals grew after a few days reaching maximum dimension of 200 x 200 x 500 µm. Crystals grown from 42% PEG400 were transferred to a 5 µl drop of soaking solution on the same cover slip (42% PEG400, 100 mM Tris-HCl pH 8.5 and 2 mM compound **11i**) and allowed to re-equilibrate with the well overnight. Crystals were frozen directly from these drops but the results were disappointing as diffraction only extended to around 10 Å. In order to improve the resolution, crystals were dehydrated by transferring the cover slips to wells containing 70% PEG400 and allowing them to equilibrate. Crystals frozen from the dehydrated drops diffracted to around 3Å on a rotating anode X-ray source. Data from crystals soaked with Compound **11i** were collected on beamline ID29 at the ESRF using 1º oscillations and a wavelength of 0.93 Å. 120 images were integrated using MOSFLM[1] and scaled using Scala[2] Scaling statistics suggested the crystal belonged to a trigonal system with cell dimensions of a = b = 108.2 Å, c = 135.3Å α = β = 90.0º, λ = 120.0º. Table 1 gives a summary of the data collection statistics. Molecular replacement was successfully used to solve the structure in spacegroup P3121 using AMORE[3]and human 11β-HSD1 (2BEL)[4] as a model. Model building and refinement were conducted using COOT[5], Refmac[6] and Buster.[7]

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7. Bricogne G.; Blanc E.; Brandl M.; Flensburg C.; Keller P.; Paciorek P.; Roversi P.; Sharff A.; Smart O.; Vonrhein C.; Womack T (2010). BUSTER version 2.11.1 Cambridge, United Kingdom: Global Phasing Ltd.

Table 1. Crystallographic Data Collection and Refinement Statistics

Space Group:	P3121	Cell Parameters:	108.2Å 108.2 Å 135.3Å 90.0º 90.0º 120.0º
Number Observations:	87158	Number Unique Reflections:	24156
Low Resolution:	54.88Å	Outer Shell Low Resolution:	2.8Å
High Resolution:	2.73Å	Outer Shell High Resolution:	2.73Å
Overall Redundancy:	3.6	Outer Shell Redundancy:	3.7
Overall I/Sigma:	13.5	Outer Shell I/Sigma:	1.8

Overall Completeness:	97.6	Outer Shell Completeness:	99.1
Overall R-merge[a]:	0.056	Outer Shell R-merge[a]:	0.571
Resolution range	20.0-2.73	Rwork/Rfree	0.198/0.223
No of refined atoms	4119	Rms deviations Bonds (A) Angles(degrees) Torsions (degrees)	 0.01 1.28 20.2
Ramachandran Preferred Regions Allowed Outliers	 484 (94.1) 26 (5.1) 4 (0.8)	Average B factors (A^2)	97.6

[a] R-merge = $\sum|(I_{hkl}) - \langle I\rangle|/(\sum(I_{hki})$ where I_{hkl} is the integrated intensity of a given reflection.
[b] Rwork = $\sum_h|F_o(h) - F_c(h)|/\sum_h|F_o(h)|$ where $F_o(h)$ and $F_c(h)$ are observed and calculated structure factors.

Procedures for determination of physicochemical properties:
$logD_{7.4}$, plasma-protein binding and solubility measurements were made as described in; Buttar, D.; Colclough, N.; Gerhardt, S.; MacFaul, P. A.; Phillips, S. D.; Plowright, A.; Whittamore, P.; Tam, K.; Maskos, K.; Steinbacher, S.; Steuber, H. A. Combined spectroscopic and crystallographic approach to probing drug–human serum albumin interactions. *Bioorg. Med. Chem.* **2010**, *18,* 7486-7496.

$logD_{7.4}$:
$LogD_{7.4}$ measurements were made using a shake-flask method where the extent of partitioning between pH 7.4 buffer and octanol was measured. Compounds were dissolved in a known volume buffer, and following the addition of a known amount of octanol, the solutions were shaken for 30 min. Following centrifugation, analysis of the aqueous layer was performed by LC–UV to quantify the amount of compound in solution and then compared to analysis of the compound in solution before the addition of octanol to calculate the partitioning coefficient, $D_{7.4}$.

Solubility:
Assessments of aqueous solubility were made after an incubation of 24 h in pH 7.4 phosphate buffer. After centrifugation, analysis of the supernatant liquid was performed by LC–UV to quantify the amount of compound in solution.

Protein binding strength *via* equilibrium dialysis:
Dialysis membranes (Spectra/Por 2, 12–14 kDa molecular weight cut-off, 47 mm diameter, Spectrum Laboratories) were prepared for use by washing with distiled water and subsequent soaking in phosphate buffer (pH 7.4). Membranes were then blotted dry and placed between two 1 mL Teflon dialysis half-cells (Braun ScienceTec, Les Ulis, France). Each half-cell was filled individually with 1 mL of protein solution containing the compound of interest, while the corresponding half-cell was filled with 1 mL of isotonic phosphate buffer. Dialysis units were immersed in a 37 °C temperature-controlled water bath and rotated at 30 rpm for 18–19 h using a Dianorm apparatus (Braun ScienceTec). After this period, samples from both the

half-cell containing buffer (protein free) and the half-cell containing protein were submitted for HPLC analysis using an Agilent 1100 series HPLC with a 110 binary pump and a UV diode ray detector. Acquisition and integration were carried out using Chemstation software (Agilent Technologies) version A.06.03 with relevant customised macro software. Integration of the subsequent chromatograms, are used to calculate the concentration of drug in the protein containing solution (Dp) and in the protein-free solutions (Df), which are then used to derive the binding constant for the test compound (K_1) assuming a 1:1 binding model as shown in Eq. 1 where the compound can only bind to a single site on the protein molecule. This is expressed mathematically in Eq. 2 where D and Df are the total and free drug concentrations, respectively, and Pr is the total protein concentration.

$$D + P \overset{K_1}{\rightleftharpoons} DP \qquad \text{Eq. 1}$$

$$D = (D_f + D_p) = \frac{K_1 \cdot D_f \cdot Pr}{1 + K_1 \cdot D_f} + D_f \qquad \text{Eq. 2}$$